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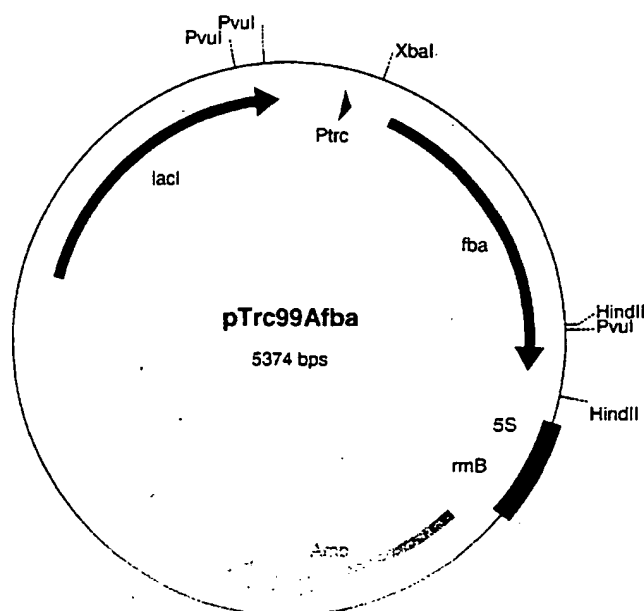
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(54) Title: **PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY**



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(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

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**Process for the Preparation of L-Amino Acids using Strains  
of the Enterobacteriaceae Family**

Field of the Invention

This invention relates to a process for the preparation of  
5 L-amino acids, in particular L-threonine, using strains of  
the Enterobacteriaceae family in which at least the fba  
gene is enhanced.

Prior Art

L-Amino acids, in particular L-threonine, are used in human  
10 medicine and in the pharmaceuticals industry, in the  
foodstuffs industry and very particularly in animal  
nutrition.

It is known to prepare L-amino acids by fermentation of  
strains of Enterobacteriaceae, in particular Escherichia  
15 coli (E. coli) and Serratia marcescens. Because of their  
great importance, work is constantly being undertaken to  
improve the preparation processes. Improvements to the  
process can relate to fermentation measures, such as e.g.  
stirring and supply of oxygen, or the composition of the  
20 nutrient media, such as e.g. the sugar concentration during  
the fermentation, or the working up to the product form, by  
e.g. ion exchange chromatography, or the intrinsic output  
properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are  
25 used to improve the output properties of these  
microorganisms. Strains which are resistant to  
antimetabolites, such as e.g. the threonine analogue  $\alpha$ -  
amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for  
metabolites of regulatory importance and produce L-amino  
30 acid, such as e.g. L-threonine, are obtained in this  
manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

#### Object of the invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Description of the invention

The invention provides a process for the preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which the nucleotide sequence which codes for the fba gene is enhanced.

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 5 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

The process is characterized in that the following steps are carried out:

- 10 a) fermentation of microorganisms of the Enterobacteriaceae family in which the fba gene is enhanced,
  - b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of 15 the Enterobacteriaceae family, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.
- 20 The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen 25 from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.
- 30 Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

- Escherichia coli TF427  
Escherichia coli H4578  
Escherichia coli KY10935  
Escherichia coli VNIIgenetika MG442  
5 Escherichia coli VNIIgenetika M1  
Escherichia coli VNIIgenetika 472T23  
Escherichia coli BKIIM B-3996  
Escherichia coli kat 13  
Escherichia coli KCCM-10132.
- 10 Suitable L-threonine-producing strains of the genus  
Serratia, in particular of the species Serratia marcescens,  
are, for example
- Serratia marcescens HNr21  
Serratia marcescens TLr156  
15 Serratia marcescens T2000.
- Strains from the Enterobacteriaceae family which produce L-  
threonine preferably have, inter alia, one or more genetic  
or phenotypic features chosen from the group consisting of:  
resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to  
20 thialysine, resistance to ethionine, resistance to  $\alpha$ -  
methylserine, resistance to diaminosuccinic acid,  
resistance to  $\alpha$ -aminobutyric acid, resistance to  
borrelidin, resistance to rifampicin, resistance to valine  
analogues, such as, for example, valine hydroxamate,  
25 resistance to purine analogues, such as, for example, 6-  
dimethylaminopurine, a need for L-methionine, optionally a  
partial and compensable need for L-isoleucine, a need for  
meso-diaminopimelic acid, auxotrophy in respect of  
threonine-containing dipeptides, resistance to L-threonine,  
30 resistance to L-homoserine, resistance to L-lysine,  
resistance to L-methionine, resistance to L-glutamic acid,  
resistance to L-aspartate, resistance to L-leucine,  
resistance to L-phenylalanine, resistance to L-serine,  
resistance to L-cysteine, resistance to L-valine,  
35 sensitivity to fluoropyruvate, defective threonine

- dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement  
5 of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form,  
10 enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.
- 15 It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after enhancement, in particular over-expression, of the fba gene.
- 20 The use of endogenous genes is in general preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

The nucleotide sequences of the genes of Escherichia coli  
25 belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453-1462 (1997)).

The following information on the fba gene is known, inter alia, from the prior art:

- 30 Description: Fructose biphosphate aldolase (class II)  
EC No.: 4.1.2.13

Reference: Alefounder et al.; Biochemical Journal  
257(2): 529-34 (1989) Zgiby et al.;  
European Journal of Biochemistry 267(6):  
1858-1868 (2000) Baldwin et al.;  
5 Biochemical Journal 169(3): 633-641 (1978)  
Accession No.: AE000376  
Alternative gene names: fda, ald

The nucleic acid sequences can be found in the databanks of  
the National Center for Biotechnology Information (NCBI) of  
10 the National Library of Medicine (Bethesda, MD, USA), the  
nucleotide sequence databank of the European Molecular  
Biologies Laboratories (EMBL, Heidelberg, Germany or  
Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima,  
Japan).

15 Alleles of the fba gene which result from the degeneracy of  
the genetic code or due to "sense mutations" of neutral  
function can furthermore be used.

To achieve an enhancement, for example, expression of the  
genes or the catalytic properties of the proteins can be  
20 increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the  
corresponding genes can be increased, or the promoter and  
regulation region or the ribosome binding site upstream of  
the structural gene can be mutated. Expression cassettes  
25 which are incorporated upstream of the structural gene act  
in the same way. By inducible promoters, it is  
additionally possible to increase the expression in the  
course of fermentative L-threonine production. The  
expression is likewise improved by measures to prolong the  
30 life of the m-RNA. Furthermore, the enzyme activity is  
also enhanced by preventing the degradation of the enzyme  
protein. The genes or gene constructs can either be  
present in plasmids with a varying number of copies, or can  
be integrated and amplified in the chromosome.

Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert,  
5 inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80:  
10 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and  
15 Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)),  
20 pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80(21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, wherein the plasmid vector carries at least one nucleotide sequence  
25 which codes for the fba gene, can be employed in a process according to the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology  
30 171: 4617 - 4622 (1989)), conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the L-threonine biosynthetic pathway or enzymes of



anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in addition to the enhancement of the fba gene.

Thus, for example, one or more of the genes chosen from the  
5 group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene which codes for pyruvate carboxylase (DE-A-  
10 19 831 609),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene which codes for phosphoenol pyruvate  
15 carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- 20 • the mgo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes  
25 for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),

- the dps gene which codes for the global regulator Dps (Genes & Development 6(12B): 2646-2654 (1992), Accession No. AE000183),
- 5 • the lrp gene which codes for the regulator of the leucine Lrp regulon and high-affinity transport systems of branched-chain amino acids (Journal of Biological Chemistry 266(17): 10768-10774 (1991), Accession No. AE000191),
- 10 • the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212(2): 199-202 (1988), Accession No. AE000222),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994), Accession No. AE000172),
- 15 • the ptsG gene which codes for the glucose-specific IIBC component of the phosphotransferase system PTS (Journal of Biological Chemistry 261(35): 16398-16403 (1986), Accession No. AE000210),
- 20 • the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 25 • the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- 30 • the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),

- the mopB gene which codes for chaperone GroES (Journal of Biological Chemistry 261(26): 12414-12419 (1986), Accession No. AE000487),
- 5 • the ahpC gene of the ahpCF operon which codes for the small subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),
- 10 • the ahpF gene of the ahpCF operon which codes for the large subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),

can be enhanced, in particular over-expressed.

The use of endogenous genes is in general preferred.

15 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the fba gene, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- 20 • the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- 25 • the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenolpyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- 30

- the poxB gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),
- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- 5 • the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor  
10 (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene, and
- the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,
- 15 to be attenuated, in particular eliminated or for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are  
20 coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

- 25 By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the  
30 starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the fba gene, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing  
5 Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed  
10 batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction  
15 to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of  
20 the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

25 Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid,  
30 alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by  
5 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine,  
10 L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in  
15 Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory  
20 Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America 86: 2172-2175 (1989)).

25 The incubation temperature for the preparation of strains and transformants is 37°C.

#### Example 1

Construction of the expression plasmid pTrc99Afba

The fba gene from E. coli K12 is amplified using the  
30 polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the fba gene in E. coli K12 MG1685 (Accession Number

AE000376, Blattner et al. (Science 277, 1453 - 1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

fba1: 5' - CGACGATACAGGACAAGAGAC - 3' (SEQ ID No. 1)

5 fba2: 5' - GCAGAAAGGAATATCTTACAG - 3' (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 1100 bp in size can be amplified  
10 with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is  
15 PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturer's instructions and transformed in the E. coli strain TOP10. Selection for plasmid-carrying cells is carried out on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the  
20 plasmid DNA, the vector pCR-Blunt II-TOPO-fba is cleaved with the restriction enzymes SpeI and XbaI and, after separation in 0.8% agarose gel with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), the fba fragment is isolated. The vector pTrc99A (Pharmacia  
25 Biotech, Uppsala, Sweden) is cleaved with the enzyme XbaI and ligated with the isolated fba fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin  
30 are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HindIII and PvuII. The plasmid is called pTrc99Afba (figure 1).



Example 2

Preparation of L-threonine with the strain MG442/pTrc99Afba

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited  
5 as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99Afba described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar  
10 with 50 µg/ml ampicillin. The strains MG442/pTrc99Afba and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l  
15 glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  
20  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  
25  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner,  
30 but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction  
5 with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99Afba	5.9	2.6

Brief Description of the Figure:

- 10 • Figure 1: Map of the plasmid pTrc99Afba containing the fba gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- 15 • Amp: Ampicillin resistance gene
- lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- fba: Coding region of the fba gene
- 20 • 5S: 5S rRNA region
- rrnBT: rRNA terminator region

The abbreviations for the restriction enzymes have the following meaning

- HindIII: Restriction endonuclease from *Haemophilus influenzae* R<sub>d</sub>
- 5 • PvuII: Restriction endonuclease from *Proteus vulgaris*
- SpeI: Restriction endonuclease from *Sphaerotilus* species ATCC 13923
- XbaI: Restriction endonuclease from *Xanthomonas campestris*

**What is claimed is:**

1. Process for the preparation of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the fba gene or the nucleotide sequence which codes for this is enhanced, in particular over-expressed,
  - 10 b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof
  - 15 optionally remaining in the product.
2. Process according to claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 20 3. Process according to claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
4. Process according to claim 1, wherein the expression of
- 25 the polynucleotide which codes for the fba gene is increased.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (protein) for which the polynucleotide fba codes are
- 30 improved or increased.

6. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 5
- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene which codes for pyruvate carboxylase,
- 10
- 6.3 the pps gene which codes for phosphoenolpyruvate synthase,
- 6.4 the ppc gene which codes for phosphoenolpyruvate carboxylase,
- 15
- 6.5 the pntA and pntB genes which code for transhydrogenase,
- 6.6 the rhtB gene which imparts homoserine resistance,
- 6.7 the mgo gene which codes for malate:quinone oxidoreductase,
- 20
- 6.8 the rhtC gene which imparts threonine resistance,
- 6.9 the thrE gene which codes for the threonine export protein,
- 25
- 6.10 the gdhA gene which codes for glutamate dehydrogenase,
- 6.11 the dps gene which codes for the global regulator Dps,

- 5      6.12      the lrp gene which codes for the regulator of  
         the leucine Lrp regulon,
- 6.13      the hns gene which codes for the DNA-binding  
         protein HLP-II
- 10      6.14      the pgm gene which codes for  
         phosphoglucomutase,
- 6.15      the ptsG gene which codes for the glucose-  
         specific IIBC component,
- 6.16      the ptsH gene which codes for the  
         phosphohistidine protein hexose  
         phosphotransferase,
- 6.17      the ptsI gene which codes for enzyme I of the  
         phosphotransferase system,
- 15      6.18      the crr gene which codes for the glucose-  
         specific IIA component,
- 6.19      the mopB gene which codes for chaperone GroES,
- 6.20      the ahpC gene which codes for the small sub-  
         unit of alkyl hydroperoxide reductase,
- 20      6.21      the ahpF gene which codes for the large sub-  
         unit of alkyl hydroperoxide reductase,

is or are enhanced, in particular over-expressed, are fermented.

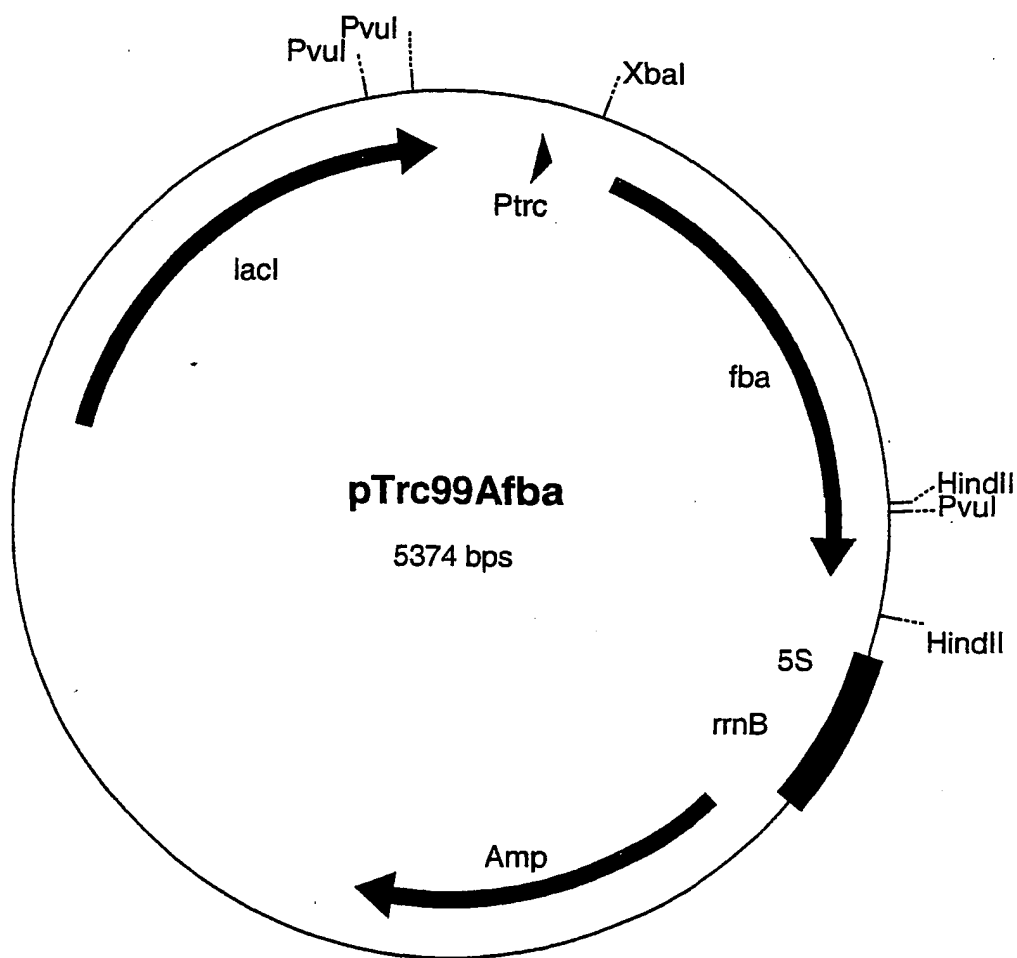
- 25      7.      Process according to claim 1, wherein, for the  
         preparation of L-amino acids, microorganisms of the  
         Enterobacteriaceae family in which in addition at the  
         same time one or more of the genes chosen from the  
         group consisting of:

- 7.1      the tdh gene which codes for threonine  
         dehydratase,

- 7.2 the mdh gene which codes for malate dehydrogenase,
- 7.3 the gene product of the open reading frame (orf) yjfa,
- 5 7.4 the gene product of the open reading frame (orf) ytfP,
- 7.5 the pckA gene which codes for phosphoenol pyruvate carboxykinase,
- 7.6 the poxB gene which codes for pyruvate oxidase,
- 10 7.7 the aceA gene which codes for isocitrate lyase,
- 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
- 7.9 the fruR gene which codes for the fructose repressor,
- 15 7.10 the rpoS gene which codes for the sigma<sup>38</sup> factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Figure 1:





## SEQUENCE PROTOCOL

5 <110> Degussa AG  
<120> Process for the preparation of L-amino acids using strains of the Enterobacteriaceae family  
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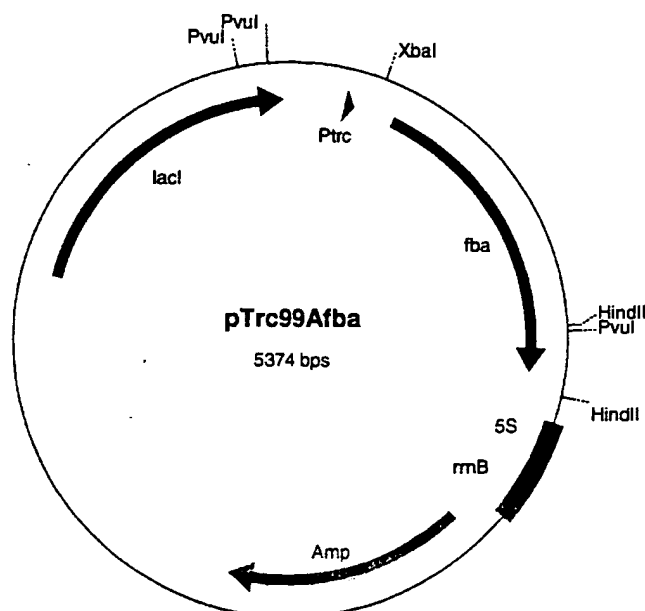
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY WITH ENHANCED FBA EXPRESSION



WO 03/004664 A3

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

# INTERNATIONAL SEARCH REPORT

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**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N15/31 C12P13/08

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**B. FIELDS SEARCHED**

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Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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A	--- EP 0 994 190 A (AJINOMOTO KK) 19 April 2000 (2000-04-19) the whole document	1-7
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## INTERNATIONAL SEARCH REPORT

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PCT/EP 02/06564

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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International Application No

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